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# Bovine growth hormone treatment increased IGF-I in circulation and induced the production of a specific immune response in rainbow trout (*Oncorhynchus mykiss*)

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## Abstract

Recombinant bovine growth hormone (rbGH) increases growth rates in rainbow trout, *Oncorhynchus mykiss*, and this response is thought to be under the control of the GH–IGF axis, as it is in mammals. However, the mechanisms regulating fish muscle growth are poorly understood. Therefore, an experiment was conducted to examine the effects of rbGH on growth-related hormones in rainbow trout. Rainbow trout ($550 \pm 10$ g) received an intraperitoneal injection of rbGH ($120 \mu g \ g^{-1}$ BW) or vehicle on days 0 and 21. Blood samples were collected on days 0, 0.5, 1, 3, 7, and 28 and assayed for rainbow trout growth hormone (tGH), rbGH, and IGF-I. As expected, rbGH levels increased ($P<0.05$) in circulation 12 h after treatment and continued to increase ($P<0.05$) from day 0 to day 7 after treatment. Unexpectedly, levels of rbGH detected at day 28, 7 days after the second injection, were lower than those at day 7. Corresponding to this observation, anti-rbGH antibodies were detectable in serum from treated fish at day 28, but not at day 7. We suggest that the low levels of rbGH detected at day 28 were due to increased clearance of rbGH caused by the anti-rbGH antibodies. Treated fish also exhibited increased serum IGF-I levels ($P<0.01$) following rbGH injection, while endogenous tGH did not change ($P=0.28$). These results suggest that the endogenous negative feedback control loop described in mammals is not activated by rbGH in rainbow trout, as tGH was unaffected by increased circulating rbGH and IGF-I. However, consistent with previous reports, rbGH does increase circulating IGF-I over time and rbGH is detectable throughout the 3-week injection period. To our knowledge, this is the first report demonstrating specific antibody production following an exogenous rbGH injection in fish.
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## 1. Introduction

Many researchers have demonstrated that treatment with growth hormone (GH) increased (averaging 40–60%) growth rates and improved feed conversion ratios in several fish species (Pickford and Thompson 1948; Swift 1954; Higgs et al., 1975, 1976; Markert et al., 1977; Kayes 1978; Gill et al., 1985; Agellon et al., 1988a,b; Schulte et al., 1989, Garber et al., 1995).

However, it is not well documented how bovine GH affects endogenous GH and IGF-I in trout, and whether exogenous rbGH elicits an immune response resulting in the production of antibodies specific to rbGH.

It is still only speculation that the GH–IGF axis in fish functions similarly to that in mammals. Recent reports of extrapituitary GH production (Yang et al., 1999), characterization of two GH isoforms (Agellon et al., 1988a), and differences in GH release patterns in teleosts (Peterson et al., 2003) have established the need to carefully examine the GH–IGF axis in fish. The increasing interest in manipulating growth of commercially important fish species has led to the need to understand the physiological effects of sustained-release rbGH treatment outside of increased growth rates. Given the differences in regulation and production of GH between mammals and fish, and the relatively low homology between bovine and trout GH (40%), it is possible that treatment with bovine GH might regulate endogenous GH differently than the well-characterized negative feedback loop that regulates endogenous GH in vertebrates (see review, Norman and Litwack, 1997). Because of the relatively low amino acid sequence homology of bovine GH, it is likely to stimulate an immune response and result in the production of specific antibodies recognizing rbGH, creating a unique and more complex growth regulatory system.

The aim of this study was to evaluate the effects of sustained-release rbGH (Posilac®, Monsanto) treatment on circulating trout GH and IGF-I levels in rainbow trout, as well as determine the presence of specific antibodies to rbGH in circulation following treatment. We hypothesized that treatment with rbGH would increase circulating IGF-I levels up to 2 weeks after treatment and endogenous trout GH would decrease in circulation due to negative feedback regulation of pituitary production and/or release of

GH. Due to the well-documented rbGH-induced growth responses of trout, we hypothesized that the rbGH treatment would not induce a specific immune response and, therefore, rbGH-specific antibodies would not be present in the serum of treated or control fish.

## 2. Materials and methods

### 2.1. Experimental animals

Rainbow trout (*Oncorhynchus mykiss*; $550 \pm 10$ g; male and female) were maintained at the Hagerman Fish Culture and Experiment Station (Hagerman, ID) in adjacent 144-L tanks with a single pass flow rate of 15-L $min^{-1}$, at 15 °C, and on a 14:10-h light:dark cycle. Fish were hand-fed twice daily to satiation using a commercial trout diet (Silver Cup Trout Feed, 45% CP, Murray, UT). All animal procedures were reviewed and approved by the University of Idaho Animal Care and Use Committee (Approval number 2001-41).

After an acclimation period, tanks were assigned randomly to a treatment group. Fish were either given an intraperitoneal injection of rbGH (Posilac®) in a sesame oil base ($120 \mu g\ g^{-1}$ BW, Schelling et al., 2000) or the same volume of sesame oil at days 0 and 21. Blood samples (3 mL) were collected from the caudal artery of fish on days 0, 0.5, 1, 3, 7, and 28. Blood was allowed to clot at 4 °C for 30 min, and then centrifuged at $1500 \times g$ for 15 min. Serum was then collected and stored at −20 °C for quantification of trout GH, bovine GH, anti-rbGH antibodies, and trout IGF-I.

### 2.2. Recombinant GH iodination and trout GH determination

Recombinant salmon/trout GH (GroPep, Adelaide, S.A., Australia) was iodinated as described by Peterson et al., (2003). The approximate yield of labeled rGH was 21% {total binding*(non-specific binding/total counts)} and the specific activity was estimated to be 2.8 μCi/μg rGH. Endogenous trout serum growth hormone concentrations were quantified using a previously described double antibody radioimmunoassay (RIA) (Peterson et al., 2003). The lowest detectable level of GH was 0.5 ng $mL^{-1}$ and the $ED_{50}$ was approximately 3.2 ng/mL. Briefly, the

antibody displayed 100% cross-reactivity with recombinant salmon GH, and less than 0.1% cross-reactivity with tilapia, duck, human, and bovine GH (GroPep). There was no cross-reactivity with salmon or trout gonadotropin, thyroid stimulating hormone, somatolactin, or prolactin (GroPep).

## 2.3. Bovine growth hormone determination

Recombinant bovine growth hormone levels in serum were determined using a double antibody RIA (Barnes et al., 1985) conducted by R. Michael Akers (Virginia Polytechnic Institute and State University). Briefly, purified bovine GH [bGH Cynamide 6952 (-42A)] and (USDA-bGH-I-1) were used as reference standards and for radioiodination, respectively. The primary antibody against bovine GH was raised in rabbits (NIH-GH-B18), and bound 40% of radiolabeled GH in the absence of unlabeled GH. The secondary antibody used was sheep anti-rabbit IgG. The sensitivity of the assay was 0.3 ng mL$^{-1}$, and the intra-assay coefficient of variation was 9%.

## 2.4. Insulin-like growth factor-I determination

Serum insulin-like growth factor-I levels were measured using a homologous RIA kit (GroPep, Adelaide, S.A., Australia). The limit of detection for this assay was 0.15 ng mL$^{-1}$ IGF-I. The intra-assay coefficient of variation was 4.5%. This assay cross-reacts with trout, barramundi, salmon, tuna, and tilapia IGF-I (GroPep).

## 2.5. Anti-rbGH antibody determination

Presence of trout antibodies specific for rbGH was determined using Western blot analysis. Recombinant bGH was separated using SDS–PAGE under reducing conditions and transferred to a nitrocellulose membrane (pore size 0.2 μm; #BA83, Protran, Schleicher and Schuell, Keene, NH). Non-specific binding was blocked with 3% non-fat dry milk (NFDM) in Tw-PBS-az (phosphate-buffered saline with Tween-20 and 0.02 g sodium azide). The membrane was then incubated with serum from either treated or non-treated fish (diluted 1:600 in Tw-PBS-az containing 2% NFDM). Membranes were then incubated with a monoclonal antibody specific to trout IgM (Warr 1.14

monoclonal antibody, 1:40 dilution; DeLuca et al., 1983) and then with a goat anti-mouse IgG conjugated to alkaline phosphotase (AP; 1:1000 dilution; Bio-Rad). Antibodies against human GH, shown to cross-react with bovine GH, were added to control serum for positive control. One membrane was incubated with NFDM without serum to serve as negative control. Specific binding was detected by visualization using nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in AP buffer (12.11 g Tris free base, 5.86 g NaCl, and 1.02 g MgCl in 1 L, pH 9.5), which reacts with AP to yield a blue color. The reaction was stopped by removing the BCIP-NBT-AP buffer solution and rinsing the membrane with deionized water.

## 2.6. Data analysis

Serum tGH, rbGH, and IGF-I data were analyzed using the Mixed procedures of the Statistical Analysis Systems (SAS; Version 8.1; SAS Institute, Cary, NC) and Repeated Measures analysis. The experimental design was a split plot factorial, with treatment as the whole plot and time as the split plot. The model included treatment, time, tank, and their interactions as sources of variation. The fixed effects of the model included treatment and time, with tank as a random effect. Tank within treatment was the repeated variable in the model. Regression analysis was used to characterize treatment effects over time. Results are reported as least square means±the pooled standard error. Results were considered significant when the probability of a type I error was less than 5%.

## 3. Results

Figs. 1, 2, and 3 show mean serum trout GH, rbGH, and IGF-I concentrations, respectively, from rainbow trout bled over a 28-day period after treatment with rbGH or vehicle. As expected, rbGH levels increased ($P<0.05$) in circulation 12 h after treatment (from $0.05\pm0.6$ at day 0 to $8.22\pm1.91$ μg mL$^{-1}$ at day 0.5) and remained elevated through day 7 ($10.6\pm0.6$, $14.0\pm0.6$, and $6.96\pm0.6$ μg mL$^{-1}$ at 1, 3, and 7 days after treatment, respectively) when compared to oil-injected controls. Recombinant bGH levels decreased from day 7 to day 28 ($6.96\pm0.6$ vs. $1.89\pm0.6$ μg
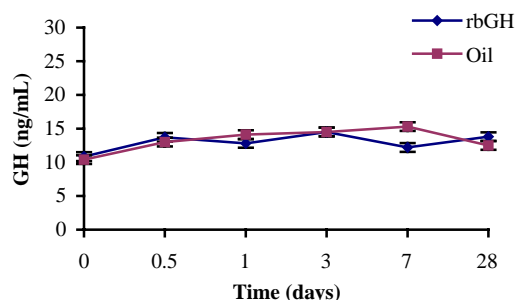
Fig. 1. Serum trout growth hormone concentrations in fish treated with either Posilac® or vehicle and bled on days 0, 0.5, 1, 3, 7, and 28. Results are least square means±pooled standard error (pSE=0.65). No treatment ($P$=0.74), time ($P$=0.55), or treatment by time interaction ($P$=0.57) was detected.

mL$^{-1}$; $P$<0.01, cubic) but remained elevated compared to controls. Serum IGF-I levels increased from 210.4±34 and 301.2±30 ng mL$^{-1}$ at day 0 to 697.1± 34 and 580.5±30 ng mL$^{-1}$ at day 0.5 and then decreased to 492.0±34 and 294±30 ng mL$^{-1}$ at day 1 in treated and controls, respectively (cubic, $P$<0.01, $y=-1104.3+1816.9x-367.2x^2-117.9x^3$, $r$=0.83). Recombinant bGH treatment increased ($P$<0.01) overall serum IGF-I levels compared to controls (577.78± 30.69 ng mL$^{-1}$ vs. 349.92±29.25 ng mL$^{-1}$). A treatment by time interaction ($P$<0.01) was detected and revealed that rbGH treatment increased (cubic, $P$<0.01, $y=-1104.3+1816.9x-367.2x^2-117.9x^3$, $r$=0.83) IGF-I levels at days 7 and 28 compared to controls. Interestingly, endogenous rainbow trout GH was not affected by treatment (12.5±0.7 ng mL$^{-1}$ vs. 13.9±0.6 ng mL$^{-1}$, $P$=0.74) and there was no treat-
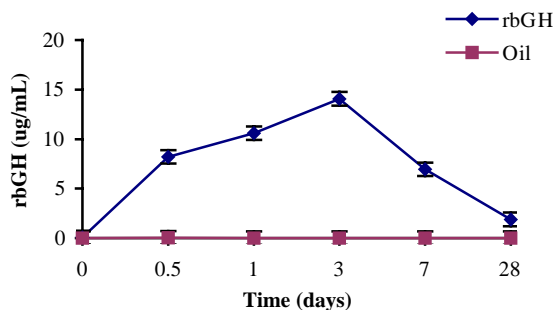


Fig. 2. Serum recombinant bovine growth hormone concentrations in fish treated with either Posilac® or vehicle and bled on days 0, 0.5, 1, 3, 7, and 28. Results are least square means±pooled standard error (pSE=0.68). Treatment ($P$<0.01), time ($P$<0.05), and treatment by time interaction ($P$<0.05) were detected.
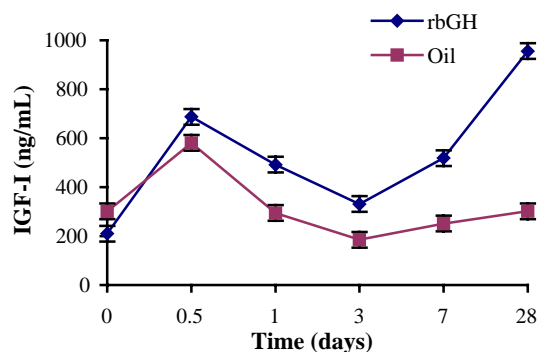


Fig. 3. Serum insulin-like growth factor-I concentrations in fish treated with either Posilac® or vehicle and bled on days 0, 0.5, 1, 3, 7, and 28. Results are least square means±pooled standard error (pSE=32). Effects of treatment ($P$<0.01), time ($P$<0.01), and treatment by time interaction ($P$<0.01) were detected.

ment by time interaction ($P$=0.57). Antibodies specifically recognizing rbGH were detected in serum from treated fish at day 28, but not at day 7 (Fig. 4). Recombinant bGH (21 kDa) was detected when incubated with positive control serum and rbGH-treated serum, indicating that rbGH-treated fish produced antibodies to rbGH.

## 4. Discussion

This study examined the effects of exogenous rbGH on circulating concentrations of endogenous rainbow trout GH, rbGH and IGF-1 as well as the production of antibodies against rbGH in fish treated with sustained-release rbGH. Results showed that exogenous rbGH increased serum IGF-1, but did not affect levels of tGH. Interestingly, levels of circulating rbGH achieved after the second injection on day 28 were lower than after the first suggesting that treated fish might have developed a specific immune response to rbGH. Analysis of serum from rbGH-treated fish at day 28 revealed the presence of antibodies to rbGH confirming this hypothesis.

Several advantages exist for sustained-release rbGH over more conventional treatment regimes, such as injection of saline-diluted rbGH. A single injection of an oil-based, sustained-release, rbGH allows for more productive utilization of a single dose because clearance rates of GH are slower for sustained-release compared to conventional prepara-
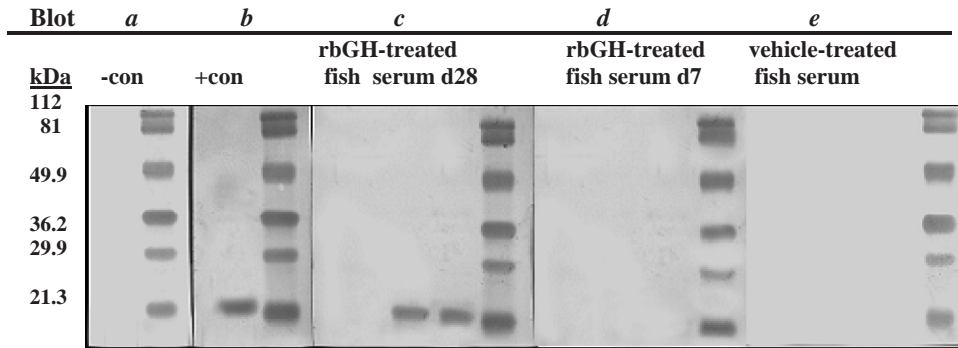
Fig. 4. Reverse Western blot analysis for rbGH antibodies in trout serum. Blots where incubated with the following: (a) sheep serum (−C; 1:600 dilution), $n$=1; (b) control trout serum (1:600 dilution) spiked with antibodies to human GH that cross react with rbGH (+C), n=1; (c) trout serum from fish treated with rbGH for 28 days (1:600 dilution of serum); (d) trout serum from fish treated with rbGH for 7 days (1:600 dilution); (e) trout serum from control fish at day 28 (1:600 dilution). For each blot, lanes were loaded as follows: blots (a) and (b)—200 ng rbGH, MW marker; blots (c, d, e)—200 ng trout GH, 100 ng rbGH, 200 ng rbGH, MW marker.

tions (McLean et al., 1997; McLean and Donaldson, 1993). Another advantage of sustained-release treatment is the reduced stress associated with reduced handling; animals would be subjected to multiple treatments using saline-based preparations.

However, with any use of a heterologous hormone, there is concern that treatment will result in activation of a specific immune response in the treated fish. Takemura (1993) demonstrated increased total IgM production in response to injections of BSA suspended in Freund's adjuvant in tilapia. Others also reported increased total IgM levels in tilapia treated with rbGH (Leedom et al., 2002), and even decreased total IgM levels following hypophysectomy in trout (Yada et al., 1999). This suggests that a link between GH and the immune system in trout is likely. Enhancement of the immune system by members of the GH/PRL family was shown in higher vertebrates (Edwards et al., 1988), and might explain changes in plasma IgM levels after Posilac® treatment. However, there are no reports of specific antibody production to rbGH in response to treatment with Posilac® in teleosts. To our knowledge, this is the first report of a specific immune response following heterologous hormone treatment in fish. It is also only speculation that the reduced rbGH levels detected in serum at day 28 (compared to the equivalent day 7 time point following the initial injection) is due to the presence of anti-rbGH antibodies causing increased clearance of rbGH from the peripheral blood. Furthermore, it is unclear if anti-idiotypic antibodies specific to rbGH were present. In the presence of anti-idiotypic antibodies, there could still be the benefit of increased growth assuming the rbGH idiotype recognizes the receptor.

Even though rbGH levels were lower at day 28 than day 7, they were still higher than non-injected controls ($1.89 \pm 0.6$ vs. $0.004 \pm 0.6$ $\mu$g mL$^{-1}$) and IGF-I levels were still elevated compared to controls. We previously reported increased IGF-I mRNA in liver tissue of these rbGH-treated fish throughout day 28 (Biga et al., 2004b). Whether these antibodies neutralized the actions of rbGH remains to be determined. It is also a possibility that the anti-rbGH antibodies might interfere with the rbGH assay, and might not affect the clearance rate in circulation. However, rbGH-treated fish do grow larger (Garber et al., 1995; Schelling et al., 2000) and liver IGF-I mRNA is still increased (Biga et al., 2004b), suggesting that any elevation of rbGH in serum promoted the growth response. Growth response may not be maximized in the presence of a specific antibody response and may result in more rapid clearance of rbGH from circulation; therefore reducing the efficacy of rbGH treatment in a commercial setting.

Perhaps one of the most interesting findings of this study was that administration of rbGH did not affect endogenous GH levels in the peripheral circulation. These results suggest that high levels of rbGH and/or IGF-I do not affect GH secretion from the pituitary and are consistent with a lack of change in endogenous GH reported in rbGH-treated tilapia (Leedom et al., 2002). It does not appear that bovine GH activates

the negative, ultra-short, feedback loop described in mammals (Grilli et al., 1997). This might be due to the nature of trout GH receptors and their ability to recognize rbGH, even though serum IGF-I levels were increased and rbGH increased growth and lowered the feed conversion ratio at this same dose (Garber et al., 1995). The low to moderate homology between bovine GH and rainbow trout GH may play an important role in binding activity and receptor signaling, which could explain the lack of ultra-short negative feedback on GH seen in this study. It is not clear if the two trout GH receptors differ in signaling mechanisms, but it is possible that they elicit different responses and that bovine GH has a higher affinity for one over the other. It is important to note that the role of GH binding proteins in trout is still unclear. It is also interesting to note, that even though bovine GH injections increased plasma IGF-I levels, endogenous trout GH levels were not affected. Increased IGF-I levels, in vitro, have been demonstrated to inhibit GH release via a long feedback loop mechanism (Blaise et al., 1995; Weil et al., 1999). It is possible that regulatory mechanisms involved in both the short and long feedback loops are modified by bovine GH, which is in contrast to trout GH which induced these feedback loops (Blaise et al., 1995; Weil et al., 1999).

Even though rbGH treatment appeared not to induce negative feedback regulation on GH, serum IGF-I levels were increased after rbGH treatment, consistent with the nature of the GH-IGF relationship described in mammals and other fish species (Silverstein et al., 2000; Kajimura et al., 2001; Biga et al., 2004b). It is well established that the growth-promoting actions of GH are mediated by IGF-I and that GH triggers the release of IGFs from the liver in mammals (see review, Florini et al., 1996). It is becoming increasingly clear that GH is the primary positive regulator of IGF-I production in teleost fishes as well (see review, Moriyama et al., 2000) and the present results are consistent with this.

Serum IGF-I levels were relatively high 12 h after treatment in both rbGH and oil-injected fish. It is unclear why IGF-I levels were not elevated ($200.0 \pm 50.0$ ng mL$^{-1}$) at the beginning of the trial. However, it is possible that the fish were not allowed enough time to acclimate to the experimental tanks and diets, may have been under short-term stress, and/or were not feeding readily at day 0. Short-term stress induces the release of ACTH and cortisol, while depressing GH and IGF-I levels (Andersen et al., 1998; Rotllant et al., 2001). In contrast, Pickering et al. (1991) demonstrated elevated GH following chronic stress, which could be restored to normal physiological levels by removing the stressor. Short-term starvation increased growth hormone levels sixfold compared to control rainbow trout (Sumpter et al., 1991). In tilapia, fasting was reported to decrease IGF-I levels after 2 weeks (Uchida et al., 2003), while other reports have demonstrated dramatic increases in IGF-I production and secretion following re-feeding in fasted trout (Chauvigne et al., 2003; Banos et al., 1999). In this study fish were fed 1 h post-treatment on day 0, therefore it is possible that the increase in circulating IGF-I at day 0.5 is due to a response of re-feeding following a short-term fasting period. Short-term stress brought about by fish relocation from outdoor-raceways to indoor-tanks could have induced short-term fasting during the brief acclimation period. Although serum IGF-I levels increased in both treated and control fish at 12 h, fish treated with GH still showed increased IGF-I levels overall and over time.

Overall results of this experiment show that rbGH increased circulating IGF-I, but did not affect endogenous trout GH, indicating that rbGH may act directly to stimulate tissues other than the liver to produce IGF-I and increase tissue sensitivity to GH. Treated fish produced antibodies specific to rbGH that were detected following the second treatment, potentially resulting in reduced rbGH levels in circulation at day 28 compared to day 7. Even though previous research demonstrated increased growth rates for up to 66 days following treatment with rbGH (Garber et al., 1995), the present results suggest that the efficiency of this growth response may diminish with time. However, previous results demonstrated increased IGF-I mRNA levels in several tissues, including the liver that had increased levels from day 0.5 through day 28, in the same rbGH treated fish (Biga et al., 2004a). Because liver is the primary site of IGF-I production in mammals, and circulating IGF-I and GH levels negatively regulate GH levels in mammals, we postulate that rbGH might act directly to increase IGF-I production in extra hepatic tissues, as well as to stimulate tissue production of GH in the same manner. We pre-

viously demonstrated rbGH effects on muscle tissue gene expression, where myostatin expression was affected differentially (Biga et al., 2004a) and IGF-I mRNA was increased in several tissues while heart IGF-IrA mRNA was decreased (Biga et al., 2004b) following rbGH treatment. The results presented here address the complexity of the GH–IGF axis and how this axis interacts with exogenous GH in rainbow trout. This study, along with previous reports, lays the groundwork for a better understanding of methods for improving fish growth for aquaculture purposes.

The proprietary formulation of rbGH used here, Posilac® is viscous at lower temperatures, which reduces the release rate of rbGH from the injection depot. In dairy cattle treated with 500 mg kg$^{-1}$ sustained-release rbGH, serum GH levels remained elevated for 12 days after treatment (Bilby et al., 1999). Results reported here indicate that rbGH was elevated for at least 7 days after initial treatment with Posilac® (120 µg g$^{-1}$ BW). Many investigators have reported varying lengths of detectable rbGH in circulation in various animals using different Posilac® doses: 66 days in tilapia treated at 1 mg g$^{-1}$ BW (Leedom et al., 2002), 140 days in coho salmon at 4.20 mg g$^{-1}$ BW (McLean et al., 1997), and 56 days in rainbow trout at 30 µg g$^{-1}$ BW (Garber et al., 1995). It is apparent that rearing temperature effects rbGH release rate from the injection depot, as rbGH detection is prolonged in rainbow trout and coho salmon (reared at 12 °C) compared with tilapia (reared at 24 °C) and cattle (average body temperature of 38.3 °C). The prolonged clearance of rbGH in rainbow trout is a major concern for the commercial application of Posilac® in the aquaculture industry. Furthermore, considerable debate surrounds the potential use of heterologous bioactive proteins in food-producing industries (Hallberg 1992).

## Acknowledgements

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